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PURIFICATION OF MOUSE MONOCLONAL IMMUNOGLOBULIN M BY ION-EXCHANGE LIQUID CHROMATOGRAPHY

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ABSTRACT

Immunoglobulin M (IgM) antibodies have been difficult to purify. By eluting with a gradient of increasing salt and decreasing pH, we succeeded in highly purifying mouse monoclonal IgM antibodies in one step on ion-exchange high performance liquid chromatography (HPLC). Two of the antibodies were specific for Clostridium difficile toxin A and toxin B and three were raised against a synthetic peptide mimicking the active site peptide of Torpedo californica 11s acetylcholinesterase. The ascitic fluids were diluted 1:10 in start buffer (20 mM Tris HCl, pH 8.5) with enough NaCl to allow solubilization of the IgM, and injected onto a diethylaminoethyl (DEAE) 5PW column, 7.5 mm x 7.5 cm, equilibrated with start buffer. Elution was as follows: (1) 10 min linear gradient to 50% start buffer, 50% limit buffer (20 mM Tris HCl, 0.3 M NaCl, pH

7.0); (2) 10 min, no change; (3) 10 min linear gradient to 100% limit buffer; (4) 20 min, no change. Flow rate was 1 ml/min. IgM eluted at 30 min, at a conductance of 20 mmho and a pH of 7.6. IgM was clearly separated from transferrin (12 min), IgG (14, 15 min) and albumin (19 min). Confirmation that the 30 min peaks contained IgM was obtained using a solid-phase enzyme-linked immunosorbent assay (ELISA) with goat anti-mouse IgM labelled with alkaline phosphatase as the detecting antibody. Immunoreactivity of the IgM-containing peaks with C. difficile toxin A was confirmed by ELISA. Purified antibody was successfully coupled to alkaline phosphatase and was used to detect C. difficile toxin A in an ELISA.

INTRODUCTION

High yields of monoclonal antibodies are obtained by injection of hybridomas into the peritoneal cavities of pristane-primed mice. The antibodies prepared in this way are, however, contaminated by host proteins, including immunoglobulins. When coupling monoclonal antibodies to enzymes for use in immunoassays, it is desirable to remove these contaminants so as to increase labelling efficiency. Among the different methods that have been tried for purification of IgM from mouse ascitic fluids are precipitation with polyethylene glycol (1), affinity chromatography (2), or fast protein liquid chromatography using anion-exchange and gel filtration columns set in series (3). In this report, we describe a rapid one-step purification on anion-exchange high performance liquid chromatography. A preliminary report of this work has been presented previously (4).

MATERIALS AND METHODS

HPLC

Ion-exchange chromatography was performed with a Waters 600 multisolvent delivery system (Milford, MA). The column used for the separation was Waters Protein Pak

DEAE 5PW, 75 x 7.5 mm I. D., equilibrated with start buffer, 20 mM Tris-HCl, pH 8.5 (20T8.5). The limit buffer was 20 mM TrisHCl, 300mM NaCl, pH 7.0 (20T7.0/0.3). Flow rate was 1 ml/min, and all work was done at ambient temperature.

Conductance Measurements

The conductance of the buffers and the fractions was measured in a conductivity meter, Radiometer Model CDM 2e (Radiometer, Copenhagen).

ELISA

IgM concentrations were measured by ELISA. Dilutions of monoclonal antibody in carbonate buffer, pH 9.6, 30 μl per well, were incubated overnight in a humidity chamber. All incubations were at room temperature. After 3 washes with running tap water, 150 μl per well of filler was added and plates were incubated for 30 min. Filler was 1% bovine serum albumin (Calbiochem, LaJolla, CA) in phosphate-buffered saline (PBS) with 1 mM NaN_3 and 1 mM EDTA (PBS-AE). Testing showed that washes were unnecessary after discarding filler, presumably because subsequent reagents were diluted in this same solution. After addition of 50 μl of detecting antibody, goat anti-mouse IgM labelled with alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:200 in filler, plates were incubated 1 hr. After 3 washes with PBS-AE, substrate was added (50 μl/well p-nitrophenylphosphate, 1 mg/ml in diethanolamine buffer) and, after 1 hr, absorbance was measured at 405 nm in a microtiter plate reader (Model EL310, Bio-Tek Instruments, Inc., Winooski, VT). Quantitation of the IgM content was determined by comparison with a standard curve; purified mouse myeloma protein, MOPC 104E (IgMg1; Organon Teknika, Malvern, PA), was bound to the solid

phase in concentrations from 250 to 0.244 μg/ml and assayed as described above.

For the ELISA to detect binding of antibody to C. difficile toxin A (5), we added 100 μl per well of toxin diluted in PBS to 13 μg/ml and dried the antigen onto the solid phase by overnight incubation. After blocking with filler (0.5% horse serum in PBS), we discarded filler and added (without an intervening wash) 100 μl of appropriate monoclonal antibody dilutions in filler (5% horse serum and 0.05% Tween 20 in PBS). After a 1 hr incubation, solutions were replaced with 100 μl per well of goat anti-mouse IgM antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD), which was incubated 1 hr, then replaced by substrate, 100 μl per well of p-nitrophenylphosphate, 1 mg/ml in diethanolamine buffer. Color development was stopped after 30 min at room temperature by addition of NaOH. All incubations were at 37°C unless otherwise noted.

Coupling of Purified Monoclonal IgM to Alkaline Phosphatase

We coupled alkaline phosphatase to purified monoclonal antibody by glutaraldehyde cross-linking. After centrifugation (12,000 x g, Brinkmann Model 5412) of 1.3 mg of a suspension of alkaline phosphatase (Sigma Chemical Co., St. Louis, MO), 0.5 mg purified IgM in 0.2 ml PBS was added to the pellet. The mixture was dialyzed against PBS, then incubated 2 hr at room temperature with 0.2% glutaraldehyde. The labelled enzyme was dialyzed against PBS and stored at 4°C.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was performed in 10% SDS slab gels (6). After heating (95°C, 5 min) in stacking gel buffer

with SDS, 2-mercaptoethanol, and 20% glycerol, 30 μl samples were added to each well in a total volume of 60 μl.

RESULTS

Salt and pH Gradients Used in Eluting Antibodies

Monoclonal IgM antibodies were purified from mouse ascitic fluids by anion-exchange HPLC. After centrifugation and filtration to remove cell debris and fibrin, 1:10 dilutions of ascites in the start buffer plus sufficient salt to solubilize the IgM were injected onto the DEAE-5PW column. The amount of salt needed to dissolve a particular antibody was determined empirically. Antibodies were eluted using concurrent gradients of increasing sodium chloride concentration and decreasing alkalinity (Table 1); as the elution proceeded, ionic strength increased and pH decreased. Figure 1 shows that the electrical conductivity (conductance) of the buffer varied linearly as the salt gradient was formed, while the change in pH was sigmoidal.

Purification of Monoclonal IgM Antibodies

Monoclonal antibodies specific for toxin A and toxin B of *C. difficile* (7) were purified from ascitic fluids

TABLE 1

Gradient Used for Elution of IgM from DEAE 5PW Column

Time (min)	% Start Buffer	% Limit Buffer
0	100	0
10	50	50
20	50	50
30	0	100
40	0	100

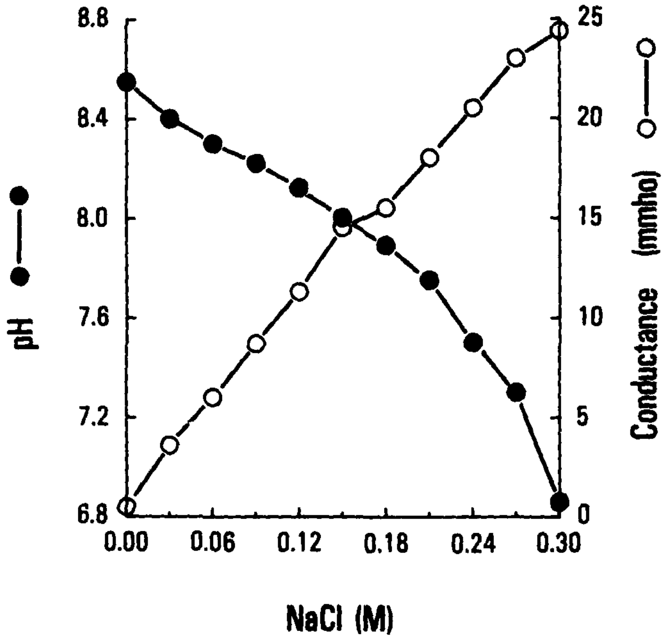

FIGURE 1. Conductance and pH of gradient used to elute IgM from DEAE-5PW column. Varying proportions of buffer 20T8.5 and buffer 20T7/0.3 were mixed to yield a range of sodium chloride concentrations. The electrical conductivity (conductance) and pH of each of these solutions was measured. Duplicate measurements were made, and the means of these determinations were plotted.

on ion-exchange HPLC using the gradient described above. The elution profiles of these IgM antibodies are shown in Figures 2 and 3. The retention time of the IgM peaks was about 30 min, clearly separated from transferrin (12 min), IgG (14, 15 min), and albumin (19 min). The identification of the various peaks was made by measuring their apparent molecular weights on gel filtration HPLC (8). The identity of the IgM peaks at approximately 30 min was confirmed with an ELISA using goat anti-mouse IgM, linked to alkaline phosphatase, as the detecting

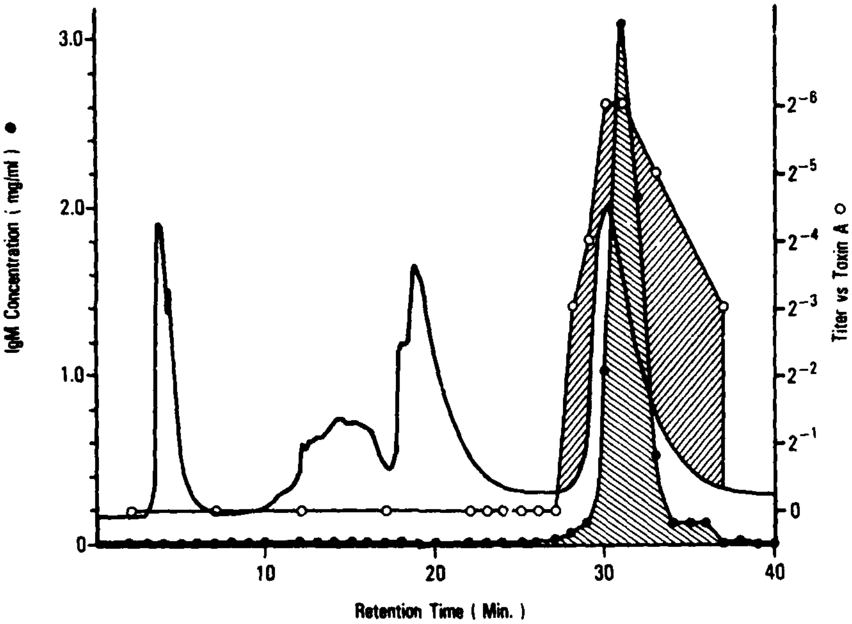

FIGURE 2. Chromatography of IgM monoclonal antibody T3-8F5 specific for C. difficile toxin A and toxin B. Monoclonal antibody T3-8F5 was purified from mouse ascitic fluid. The ascitic fluid was centrifuged briefly to remove cell debris, diluted ten-fold in equilibration buffer (20T8.5) plus enough 1 M NaCl to allow the antibody to dissolve, then filtered. A two-ml volume was injected onto an ion-exchange column (DEAE-5PW). Elution was with two successive linear gradients of increasing salt and decreasing pH (Table 1; Figure 1). IgM content of the fractions was determined by an ELISA with purified mouse myeloma protein as the standard. The binding of the fractions to highly purified C. difficile toxin A was measured in an ELISA.

antibody. For quantitation of the amount of IgM present, the ELISAs were standardized with purified mouse myeloma IgM. The immunoreactivity of the IgM fractions was confirmed in an ELISA with purified C. difficile toxin A as the antigen. Recovery of the toxin binding activity was 41% for antibody T3-8F5. The conductance at which

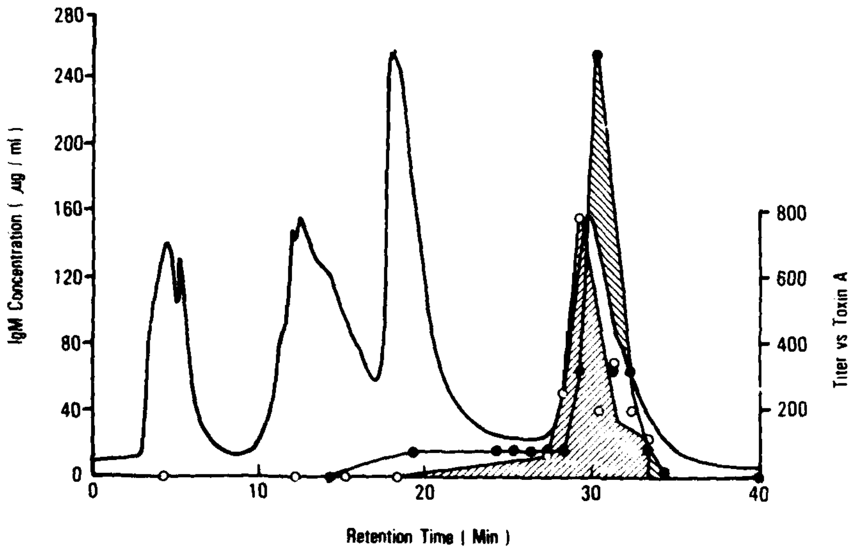

FIGURE 3. Chromatography of IgM monoclonal antibody T3-4C11 specific for C. difficile toxin A and toxin B. Monoclonal antibody T3-4C11 was purified from mouse ascitic fluids as described in the legend to Figure 2.

antibody T3-8F5 eluted from the column was 20 mmho; this corresponded to a pH of 7.6.

Analysis of the IgM peak by gel filtration HPLC suggested that there was still some dimeric mouse albumin contaminating the IgM, and the presence of mouse albumin in the IgM fraction was confirmed by ELISA and by SDS-PAGE (Figure 4).

Three IgM monoclonal antibodies raised against a synthetic peptide mimicking T. californica 11s acetylcholinesterase (M. K. Gentry, unpublished data) were purified in the same manner (Figure 5). Retention times of the IgM peaks were 27 min for each. The IgM concentration of the pooled peak fractions was determined by ELISA with mouse myeloma IgM as the standard.

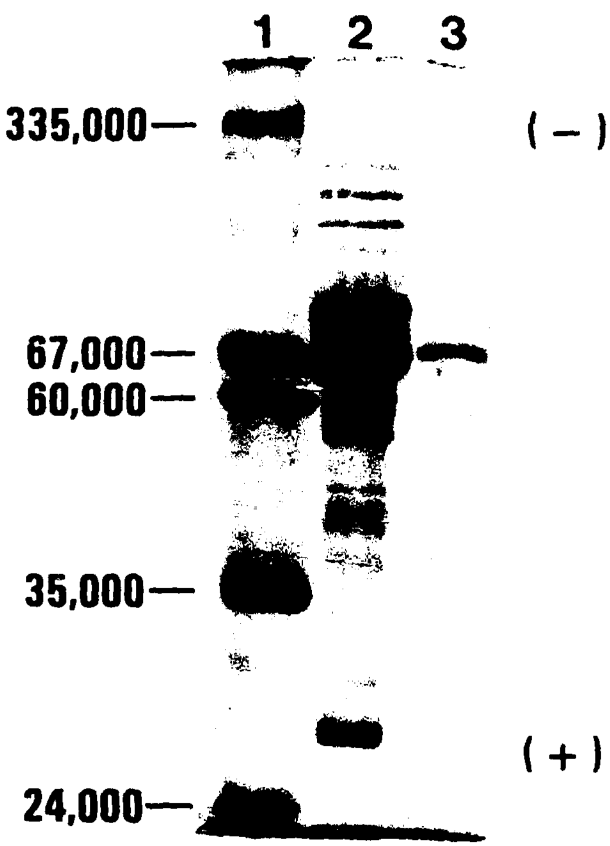

FIGURE 4. Electrophoretic characterization of IgM after purification by HPLC. After SDS-PAGE, proteins were visualized by Coomassie blue staining. Lane 1, molecular weight standards; lane 2, IgM specific for C. *difficile* toxin A and toxin B before HPLC; lane 3, pooled IgM fractions after HPLC purification.

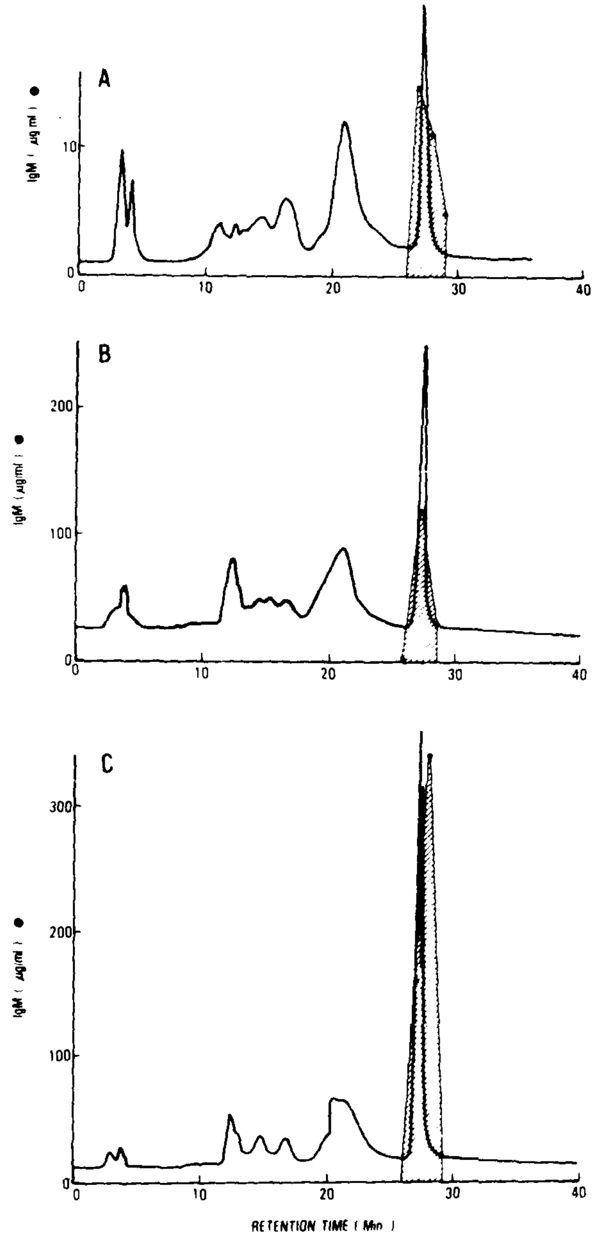

FIGURE 5. Chromatography of IgM monoclonal antibodies specific for T. californica 11s acetylcholinesterase. These IgM monoclonal antibodies were raised against a synthetic peptide mimicking the active site peptide of T. californica 11s acetylcholinesterase. Figure 5A is antibody 1B3-5; B is antibody 4A2-5; C is 14A7-9. Antibodies were purified from mouse ascitic fluids as described in the legend to Figure 2.

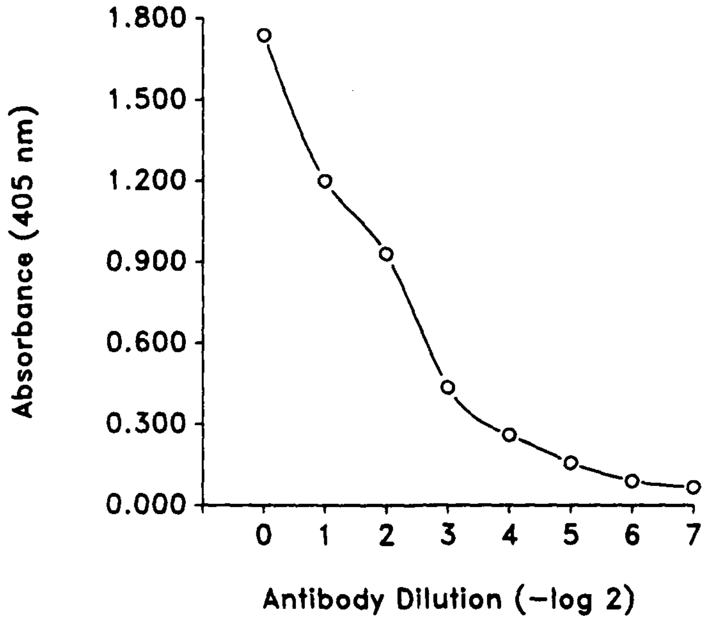

FIGURE 6. ELISA with purified IgM as the detecting antibody. Purified IgM monoclonal antibody T3-8F5 was coupled to alkaline phosphatase. Purified C. difficile toxin A was bound to the solid phase and was detected by addition of substrate after addition of the labeled T3-8F5.8

Coupling of Purified IgM Monoclonal Antibody to Alkaline Phosphatase

We coupled purified monoclonal antibody T3-8F5 to alkaline phosphatase and used it in an ELISA with C. difficile toxin A bound to the solid phase (Figure 6). Binding was 50% of maximal at a dilution of approximately 1:125.

DISCUSSION

We have succeeded in rapidly purifying IgM monoclonal antibodies. The purified antibody can be used

for enzyme coupling and other procedures, such as competitive ELISAs. As the antibody is put on the column without any prior processing, the entire purification can be completed in about one hour. There is no need to use harsh ingredients such as ammonium sulfate, acids or chaotropic agents. Lim (2) reported almost no recovery of IgM from affinity columns when eluting with acid and denaturation of IgM when eluting with KSCN.

There is some mouse albumin which copurifies with the IgM. For most procedures, this does not create a problem. It can, if necessary, be removed by further purification by gel filtration HPLC. Clezardin, et al. (3), separated IgM by Fast Protein Liquid Chromatography, following an initial anion exchange step with gel filtration. We have analyzed our antibodies on gel filtration HPLC (data not shown), but gel filtration steps are of limited value in rapid preparations because only relatively small amounts of sample can be added to these columns.

As the first step in our coupling procedure included dialysis against the reaction buffer, the IgM fractions from the column could be used directly once they were concentrated. The entire procedure, then, starting with crude mouse ascitic fluid and ending with labelled purified IgM can be completed in a 24-hr period.

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